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A RAPID METHOD FOR DETERMINING THE PERCENTAGE OF ANTIBACTERIAL --ETC(U)
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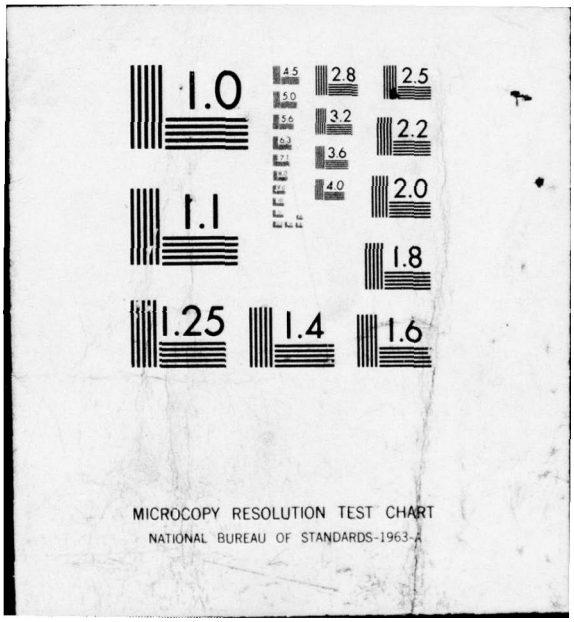
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A relatively rapid, simple direct microscopic assay of the antibacterial capability of phagocyte populations has been developed. Normal mice or mice treated with 7.6% sodium caseinate to stimulate production of peritoneal exudate cells were inoculated intraperitoneally with actively growing Vibrio parahemolyticus. Thirty minutes later phagocytes were harvested by washing the peritoneal cavity with a harvest medium that arrested phagocytosis but permitted viable intra- and extracellular vibrios to form microcolonies after incubation on

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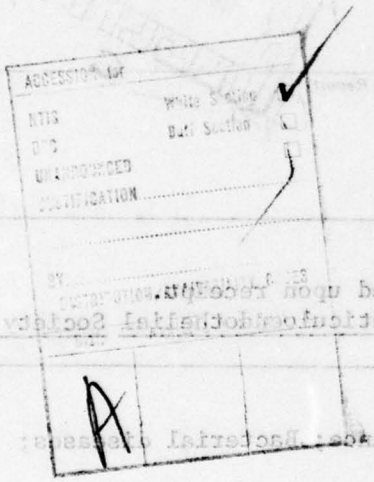
microscope slides. The antibacterial potential of the phagocyte population was determined by comparing the percentage of phagocytes with ingested vibrios before culture with the percentage of phagocytes whose intracellular V. parahemolyticus failed to replicate after incubation.

Essentially all phagocytic cells in peritoneal washings from normal mice were found to be macrophages, less than 50% of which had antibacterial activity against V. parahemolyticus. Antibacterial phagocyte populations were greatly increased following exudate induction; within 4 hr the exudates contained both neutrophils and macrophages, and essentially all phagocytizing cells had effective antibacterial capability. This technique employing an in vivo milieu for phagocyte-bacterial interaction provides optimum conditions for assays of phagocyte antibacterial capability and its modification by treatment of the donor.



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Werner A. Janssen and Harry G. Dangerfield[1]

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Short title: ASSAY OF ANTIBACTERIAL PHAGOCYTE POPULATIONS

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

A relatively rapid, simple direct microscopic assay of the antibacterial capability of phagocyte populations has been developed. Normal mice or mice treated with 7.6% sodium caseinate to stimulate production of peritoneal exudate cells were inoculated intraperitoneally with actively growing Vibrio parahemolyticus at estimated ratios of 1 to 5 vibrios per phagocyte. Thirty minutes later phagocytes were harvested by washing the peritoneal cavity with 2 ml of a harvest medium that arrested phagocytosis but permitted viable intra- and extracellular vibrios to form microcolonies after incubation for 2 hr at 37 C on microscope slides. The antibacterial potential of the phagocyte population was determined by comparing the percentage of phagocytes with ingested vibrios before culture with the percentage of phagocytes whose intracellular V. parahemolyticus failed to replicate after incubation.

Essentially all phagocytic cells in peritoneal washings from normal mice were found to be macrophages, less than 50% of which had antibacterial activity against V. parahemolyticus. Antibacterial phagocyte populations were greatly increased following exudate induction; within 1 hr the macrophage population doubled and the percentage of antibacterial macrophages was increasing; within 4 hr the exudates contained both neutrophils and macrophages, and essentially all phagocytizing cells had effective antibacterial capability. This technique employing an in vivo milieu for phagocyte-bacterial interactions provides optimum conditions for assays of phagocyte antibacterial capability and its modification by treatment of the donor.

INTRODUCTION

Nonspecific host resistance to certain infectious diseases has been related to the antibacterial capability of host phagocytes (1, 3-5). Rowley postulated that the population of phagocytes in normal animals is heterogeneous with regard to antibacterial capability, and that the level of nonspecific host resistance depends to a considerable extent upon the percentage of phagocytes capable of killing or inhibiting the growth of pathogens (5). If this is true, the relative number of antibacterial phagocytes in an animal could provide an index of its nonspecific resistance to disease, and be useful for detecting changes in nonspecific resistance which are induced by infection, immunization and various treatments. Therefore, we developed a relatively rapid, simple method for determining the percentage of phagocytes with antibacterial capability in a sample population of leukocytes. This method, a modification of a technique for direct observation of the intraphagocytic fate of bacteria (2), eliminates possible artifacts inherent in conventional methods for assessment of antibacterial activity.

MATERIALS AND METHODS

Experimental Animal. Male and female, 25-g Swiss Webster mice were used for antibacterial phagocyte population assays.

Bacterial Cultures. Vibrio parahemolyticus ATCC strain no. 1708 was employed in all studies. Liquid culture medium consisted of Bacto Brain Heart Infusion Broth (Difco Products) supplemented with 3% NaCl (BHI broth); the solidified medium (BHA) contained 1.5% Bacto agar. The lyophilized culture was transferred to 25 ml BHI in a 250-ml Ehrlenmeyer flask and the culture was incubated at 37 C for 18 hr on a reciprocal shaker (100 strokes/minute). The 18 hr growth was used to prepare the initial stock cultures on BHA slants: after the entire surface was inoculated, slants were incubated for 24 hr at 37 C; cultures were stored at 4 C and growth was transferred at monthly intervals to fresh BHA to assure viability. Cultures for inoculating mice, prepared by transferring 24-hr growth from the surface of a BHA culture into 25 ml BHI broth in a 250-ml Ehrlenmeyer flask, were incubated as described; direct microscopic counts (Petroff-Hauser method) and standard plate counts indicated that a concentration of 10^{10} vibrios/ml was achieved and all vibrios were viable. To effect estimated vibrio:phagocyte ratios of 1:1 to 5:1 for mouse inocula, cultures were diluted in BHI broth.

Peritoneal Exudate Induction. A 7.6% aqueous solution of sodium caseinate (Eastman Kodak) was adjusted to pH 7.4; after sterilization by autoclaving, the solution was stored at 4 C. The caseinate solution was heated to 39 C just before injection, and individual mice were inoculated intraperitoneally (i.p.) with 2.5 ml.

Antibacterial Phagocyte Population Assay. Individual normal or caseinate-treated mice were inoculated i.p. with 0.1 ml of an appropriate dilution of V. parahemolyticus culture, followed 30 min later by 2 ml of BHI. Each mouse was killed 1 min later by cervical dislocation and the abdomen was massaged vigorously for 30 sec. The peritoneal cavity was exposed and the wash fluid removed aseptically with a large-bore 5-ml pipette. After the total leukocyte count in the wash fluid was determined, the cells were concentrated by light centrifugation in a clinical centrifuge (Schaar Co.) for 5 min at approximately 500 r.p.m. The differential leukocyte count and percentages of neutrophils and macrophages with ingested vibrios were determined by examining a smear of concentrated leukocytes stained with Giemsa stain. A microscppe slide culture was prepared by placing a small drop of the concentrated leukocytes on a sterile microscope slide and carefully overlaying it with a sterile cover slip in such a manner as to exclude air bubbles and to obtain the thinnest preparation possible without disrupting the leukocytes. (Microscope slides and cover slips must be of highest quality, and suitable for tissue culture.) Edges of the cover slip were sealed with sterile melted vaspar (equal volumes of vasoline and paraffin). After incubation at 37 C for 2 hr, slide cultures were examined under phase illumination at 1200 X magnification to determine the percentage of neutrophils and macrophages that contained viable vibrios, i.e., intracellular microcolonies. At least 100 neutrophils and 100 macrophages were counted in each slide culture and each stained smear preparation; if the concentration of a particular type was too low to complete the count within 30 min, TFTC (too few to count) was the only data recorded for the sample. The percentage of neutrophils or macrophages which prevented intracellular replication of vibrios was calculated as follows:

$$\text{Antibacterial Index} = \frac{\text{No. with ingested vibrios} - \text{No. with viable vibrios}}{\text{No. with ingested vibrios}} \times 100$$

Finally, the actual ratio of V. parahemolyticus per peritoneal phagocyte was determined.

RESULTS

Phagocyte Population in Peritoneal Wash Fluids. The total number of phagocytic cells with percentages of neutrophils and macrophages recovered in harvest fluids from the peritoneal cavities of normal mice or mice treated by i.p. injection of sodium caseinate solution was determined (Table 1). In harvests from normal mice approximately 1.5 million potentially phagocytic cells, representing 25% of the total leukocyte population, were recoverable from the peritoneal cavity and almost all phagocytes were typical macrophages. One hour after caseinate treatment, peritoneal wash fluids contained 6 million phagocytic cells; phagocytes constituted more than half of the leukocyte population and essentially all were macrophages. By the 4th hr posttreatment the leukocyte population contained chiefly phagocytic cells, approximately 36 million in number of which most were neutrophils. In 6 to 24 hr harvests the percentage of neutrophils decreased while the percentage of macrophages increased; a maximum yield of approximately 65 million phagocytic cells was recovered at 12 hr. At 24 hr after exudate-induction approximately 85% of the peritoneal leukocyte population were phagocytic cells (2.35×10^7) with neutrophils slightly outnumbering macrophages.

Assay of Antibacterial Phagocyte Populations. In vivo antibacterial activity of phagocytes in the peritoneal cavity of normal mice or of exudate-induced mice at 1, 4 and 24 hr postinduction was compared (Table 2). Following inoculation of V. parahemolyticus, the peritoneal phagocyte

population in normal mice consisted primarily of macrophages, approximately 50% of which contained vibrios and, of these, less than 50% had antibacterial activity, i.e., vibriocidal or vibriostatic; neutrophils were rarely observed. When inoculation was administered within 1 hr following exudate induction, the percentage of macrophages containing vibrios showed little if any change, but in some mice antibacterial activity of macrophages was greatly increased; again neutrophils were too few to permit assay. In harvests from the 4-hr exudate group ingestion of vibrios by the predominating neutrophil population was quite variable, i.e., 15 to 51% of neutrophils contained vibrios, but virtually all phagocytizing neutrophils had antibacterial activity. Although macrophages harvested from the 4-hr group appeared smaller than normal with morphology suggestive of transitional forms in a rapidly changing population, nonetheless, most with ingested vibrios had antibacterial activity. By 24 hr postinduction, the percentage of phagocytizing neutrophils and macrophages had increased and essentially all phagocytizing cells had antibacterial activity.

To evaluate the possibility that i.p. destruction of vibrios might occur, peritoneal washings harvested at intervals from 2 to 30 min after inoculation with vibrios were examined microscopically. No morphologic evidence for *in vivo* destruction of either intra- or extracellular vibrios was observed in harvests from normal or exudate-induced mice.

Intraphagocytic Growth of V. parahemolyticus In Vitro. The effect of incubation time and temperature on growth of *V. parahemolyticus* within mouse peritoneal macrophages in microscope slide cultures is shown in Table 3. Slide cultures of peritoneal wash fluid from a normal mouse and from a caseinate-treated mouse were prepared 30 min after i.p. inoculation with vibrios, and incubated at 23 or 37 C. The number of vibrios within 100 macrophages

was counted in each slide culture immediately after preparation, then after 2 hr incubation at 37 C or 3 hr at 26 C, and after 24 hr. Replication of vibrios within macrophages from the normal mouse peritoneal washings was detectable within 2 hr at 37 C, and within 3 hr at 23 C. In macrophages from the caseinate-treated mouse, morphologic destruction of vibrios, i.e., pleomorphism and lysis, was observed after incubation of slide cultures for 2 hr at 37 C, while only inhibition of replication could be detected after 3 hr incubation at 23 C. After 24 hr incubation many macrophages from the normal mouse were filled with vibrios, while those from the caseinate-treated mouse were free of vibrios; however, extracellular vibrios were in such profusion in all slide cultures that resolution of phagocyte intracellular detail was difficult. Therefore, microscope slide cultures were routinely incubated at 37 C for 2 hr, permitting completion of an assay within approximately 3 hr.

Morphology of Intraphagocytic V. parahemolyticus Microcolonies. The rapid intracellular growth and microcolony morphology of V. parahemolyticus within phagocytic cells incubated at 37 C is illustrated in Figures 1-5. In microscope slide cultures prepared immediately after removal of peritoneal wash fluid from mice, phagocytes rarely contained more than 1 or 2 vibrios. Within 15 min after slide culture preparation all of the leukocytes became swollen in the culture medium required for growth of V. parahemolyticus; this alteration in the integrity of the phagocytes did not result in their lysis during the test period, but did improve visualization of the ingested bacteria. After 2 hr incubation, viable vibrios had replicated sufficiently to produce microcolonies with 16 or more organisms, many of which were actively motile so that detection of viability was not difficult. By the 24th hr of

incubation intracellular microcolonies completely filled the phagocytes, causing many to burst.

DISCUSSION

A mass of evidence indicates that phagocytes contribute significantly to nonspecific host resistance in certain infectious diseases, and to cell-mediated immune responses of the host after infection with certain facultative intracellular bacteria. Techniques for relating phagocyte antibacterial ability to nonspecific resistance or cell-mediated immunity often involve exposing phagocytes to bacteria under artificial conditions, and subsequently determining the viability of ingested organisms after disruption of the phagocytes by artificial means. Experimental variables inherent to each technique have caused divergent findings and are responsible for controversy regarding interpretation of results (2,3). We have attempted to develop an assay for phagocyte antibacterial ability which would eliminate some of these variables, and permit identification of those phagocytes that prevent intracellular growth of bacteria.

In the present assay, phagocytes are presented with test bacteria in vivo, thus permitting them to ingest and kill, or prevent growth, of the organisms within the environment of the host. V. parahemolyticus was selected as an indicator organism because: (a) its exceptionally rapid growth permits completion of an assay within 3 hr; (b) its active motility within phagocytes permits easy visualization and provides proof of intracellular survival; (c) the medium required for its growth causes swelling of phagocytes, thereby further facilitating observation of intracellular bacteria; and (d) since it is a marine organism there is little probability that the experimental animals had

prior exposure.

Assays of peritoneal washings from normal mice revealed that the phagocyte population consisted almost entirely of macrophages, of which less than 50% had antibacterial activity against V. parahemolyticus. Pretreatment of the mice by i.p. injection of sodium caseinate solution resulted in a marked increase in the total phagocyte population and in the percentage of antibacterial phagocytes in the peritoneal cavity. Neutrophils were rarely detected in peritoneal washings until 4 hr after treatment at which time they were in great abundance and almost every cell had antibacterial activity. A marked increase in the total number and percentage of macrophages with antibacterial activity occurred in some mice within 1 hr after treatment, and in all mice within 4 hr. By the 24th hr after exudate induction by caseinate treatment, there was an increase in the percentage of both neutrophils and macrophages that actively ingested V. parahemolyticus, and almost all phagocytizing cells had antibacterial activity.

This assay procedure shows promise of permitting more rapid and accurate evaluation of the antibacterial capability of phagocytic cells, and may provide a more sensitive method for detecting changes in antibacterial populations induced by various treatments and types of stress. Whether an increase in antibacterial activity against V. parahemolyticus can serve as an indicator for a concommitant increase in nonspecific resistance to lethal bacterial infections will require further investigation.

ACKNOWLEDGMENTS

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TABLE 1

Phagocyte Population in Peritoneal Wash Fluid After Exudate Induction by Sodium Caseinate

Caseinate Pre-treatment (hr)	Total No. Phagocytes x 10^{7}[a]	% Neutrophils	% Macrophages
None	0.14 (0.06 - 0.29)	3.0 (0 - 12)	22.5 (10 - 41)
1	0.62 (0.35 - 1.06)	4.0 (0 - 10)	49.5 (30 - 81)
4	3.61 (3.57 - 3.76)	90.5 (90 - 92)	4.5 (3 - 6)
6	4.84 (4.64 - 5.03)	87.0 (85 - 88)	9.0 (7 - 11)
12	6.56 (5.92 - 7.05)	75.0 (72 - 78)	18.0 (12 - 24)
24	2.35 (1.11 - 2.53)	48.0 (32 - 65)	36.0 (12 - 52)

[a] Mean value and range ( ) for 10 mice.

TABLE 2

Phagocytic and Antibacterial Activity of Mouse Peritoneal Phagocytes Harvested 30 Min After i.p. Challenge With *Vibrio parahemolyticus*

Caseinate Pre-treatment of Donor (hr)	Challenge Dose Vibrio/Phagocyte Ratio	Neutrophil[a]		Macrophage[a]	
		Phagocytic Index	Antibacterial Index	Phagocytic Index	Antibacterial Index
None	4:1	TFTC		46 (30-64)	41 (33-47)
1	2:1	TFTC		41 (37-46)	67 (30-94)
4	5:1	35 (15-51)	98 (94-100)	16 (14-16)	82 (75-88)
24	4:1	52 (43-70)	98 (96-100)	61 (48-70)	98 (96-100)

[a] Average values with range ( ) for samples from 5 mice per group; 100 neutrophils and 100 macrophages counted per sample.

Phagocytic Index = % of respective cell type with ingested vibrios.

Antibacterial Index = % phagocytizing cells in which intracellular growth of vibrios failed to occur after *in vitro* incubation.

TABLE 3

Vibrio parahemolyticus in Mouse Peritoneal Macrophages Harvested 30 Min After i.p. Challenge and Incubated In Microscope Slide Cultures

In vitro Incubation		No. Intracellular *V. parahemolyticus*/100 Macrophages	
Temperature	Hours	Normal Donor	Exudate-Induced Donor
23 C	0	162	152
23 C	3	358	134
37 C	0	186	160
37 C	2	432	0

Fig. 1-4.

Sequence of photomicrographs showing the rapidity of growth of Vibrio parahemolyticus within a mouse peritoneal macrophage in a microscope slide culture incubated at 37 C; 1200 X magnification under phase illumination. Fig. 1. 5 min after preparation of the microscope slide culture. Arrow points to a single vibrio. Fig. 2. 15 min after preparation. Note swelling of the macrophage. Fig. 3. 30 min after preparation. Note that vibrio has divided. Fig. 4. 2 hr after preparation. Note that there are now 16 vibrios. Most of the vibrios were actively motile within the confines of the macrophage cell wall.

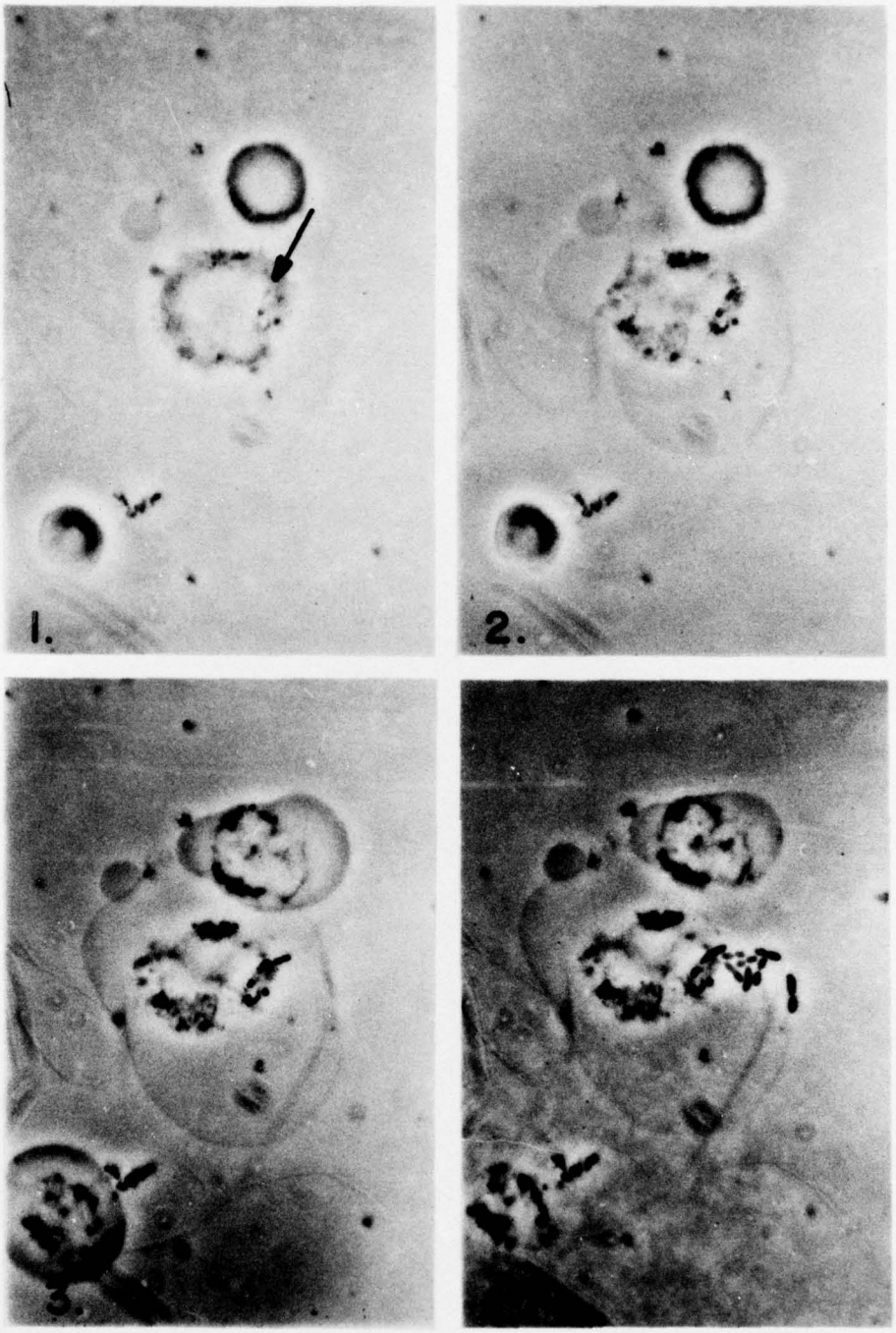
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Fig. 5.

Photomicrograph of another field 24 hr after preparation. Note that vibrios completely fill the macrophage in the center. Intracellular growth of vibrios cause many phagocytes to burst.